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PRINCIPAL INVESTIGATOR: Ginette Serrero, Ph.D.

CONTRACTING ORGANIZATION: University of Maryland, Baltimore
Baltimore, Maryland 21201-1627

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Ginette Serrero, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Maryland, Baltimore
Baltimore, Maryland 21201-1627

E-Mail: gserrero@rx.umaryland.edu

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13. ABSTRACT ***(Maximum 200 Words)***
The goal of this study was to examine the expression in human breast cancer biopsies of a novel growth factor, called PC-cell derived growth factor (PCDGF) characterized in our laboratory, and examine correlation of PCDGF expression with clinical parameters and with known prognosis markers of breast cancer. Previous biological studies with human breast cancer cell lines have indicated the role of PCDGF in the tumorigenicity of breast carcinoma and identified it as a molecular target of estrogen-independence and tamoxifen resistance. Here, wave conducted a study with 206 paraffin embedded human breast cancer biopsies and measured PCDGF expression by immunohistochemistry using an anti-PCDGF developed in our laboratory. We show that PCDGF is not expressed in normal epithelium and in benign tumors. PCDGF was preferentially expressed in in situ and invasive ductal carcinoma where its expression correlated with tumor grade. PCDGF expression significantly correlated with steroid receptor status, high proliferation index (Ki-67) and p53 expression. In contrast PCDGF expression was independent of erbB2 expression. These studies have provided novel information on the expression of a novel autocrine growth factor in human breast cancer biopsies. Future studies are proposed to examine the correlation of PCDGF expression with response to therapy in advanced breast carcinoma

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RESEARCH TECHNICAL REPORTING
Ginette Serrero, Ph.D. Principal Investigator

INTRODUCTION:

The goal of this proposal is to examine if the expression in human breast cancer biopsies of a novel growth factor, characterized in our laboratory, can be used as an early prognostic factor for estrogen-independence. The critical phase in the diagnosis and therapy of breast cancer is to determine whether a tumor is estrogen responsive or has progressed to estrogen-independence and tamoxifen-resistance. This analysis is important since it will determine if a tumor will be responsive or not to anti-estrogen therapy. Measuring estrogen receptor (ER) and progesterone receptor (PR) expression in the tumor biopsies is done for this purpose. We have cloned a novel tumorigenic growth factor called PC-Cell Derived Growth Factor (PCDGF, granulin precursor) (1) expressed in human breast carcinoma. In ER^+ cells, PCDGF expression is stimulated by estradiol and inhibited by tamoxifen (2). In ER^+ cells, PCDGF mediated estrogen mitogenic activity and overexpression of PCDGF rendered the ER^+ cells estrogen-independent for growth and tamoxifen-resistant (4) while they remained positive for ER and PR. In ER^- cells, PCDGF is overexpressed. Inhibition of PCDGF expression in ER- cells led to a 90% inhibition of tumor incidence and growth (3). These results indicated the role of PCDGF in the tumorigenicity of breast carcinoma and identified it as a molecular target of estrogen-independence and tamoxifen resistance that precedes changes in the ER/PR status of the tumors. To test this hypothesis, we have proposed in this concept award to examine the level of expression of PCDGF in paraffin embed breast cancer biopsies and correlate it with clinical parameters as well as with the expression of known prognostic markers of breast cancer.

BODY:

This annual report concerns the studies carried out under a one year Concept Award. The studies supported by this Concept Award have focused on investigating the expression of PCDGF in human breast cancer biopsies to determine whether increase of PCDGF expression correlates with advanced stages of the disease and examine whether PCDGF can be used as a prognostic biomarker for the disease. The approved statement of work and experimental protocol consisted of examining PCDGF expression in paraffin embedded breast tumor biopsies by immunohistochemistry using an anti-PCDGF antibody developed in our laboratory. Even though our original proposal was to limit our study to examining 30 samples, we have extended our studies to 206 archival samples obtained from the surgical pathology archives of the UM Cancer Center and from the Brest Cancer Tumor Bank from the University of Manitoba in Canada. These samples were provided with clinical parameters such as tumor type and grade and lymph node status. Analysis of the staining and pathological interpretation of the data was carried out in collaboration with O. Ioffe, MD, Breast Pathologist. In addition to measuring PCDGF expression, we also measured expression of estrogen receptor (ER) and progesterone receptor (PR) for the samples for which this information was not provided (UMCC archive biopsies) as well as measuring other markers known that their prognostic value such as p53, erbB2 and the proliferation index Ki67.
The results of the study are summarized below.

The study on the 206 human breast lesions has shown that PCDGF staining is observed in breast carcinoma whereas it is negative in benign breast epithelium (table1).

Table 1. Results of PCDGF immunostaining in breast biopsy samples

Diagnosis	# of cases	PCDGF staining		
		Negative	Weak (1+)	Moderate/Strong (2+/3+)
Benign	26	25 (96%)	1 (4%)	0
DCIS	27	9 (33%)	8 (30%)	10 (37%)
LCIS	12	11 (92%)	1 (8%)	0
IDC	124	25 (20.2%)	48 (38.8%)	51 (41%)
ILC	17	8 (47%)	6 (35%)	3 (18%)

IDC – invasive ductal carcinoma, ILC – invasive lobular carcinoma, DCIS – ductal carcinoma in situ, LCIS – lobular carcinoma in situ. The differences in PCDGF expression between benign/LCIS, and intraductal/invasive carcinomas are statistically significant ($p<0.05$).

Analysis of various types of breast cancer (table1) indicated that PCDGF was predominantly expressed in invasive ductal carcinoma rather than in invasive lobular carcinoma. 80% of invasive ductal carcinomas were positive for PCDGF with 41% of cases staining with at least 2+ intensity. In contrast, 47% of invasive lobular carcinomas were negative for PCDGF, and only 18% of positive tumors showed at least 2+ reactivity. No PCDGF staining was observed in lobular carcinoma in situ. Ductal carcinoma in situ expressed PCDGF in 66% of the cases, and this expression correlated strongly with the nuclear grade of DCIS. Similar correlation was observed between the degree of PCDGF expression and histological grade of invasive ductal carcinoma. Correlation studies were carried out in invasive cancers between PCDGF staining intensity and c-erbB-2, estrogen (ER), progesterone (PR) and p53 expression, and the proliferation rate expressed by Ki-67 index. The latter index was proportional to the degree of PCDGF expression in the tumor cells, with the average Ki-67 index of PCDGF-negative/weakly positive tumors (30.3) significantly lower than that of strongly PCDGF-positive carcinomas (48.8, $p<0.05$). PCDGF was expressed in both ER/PR-positive and negative tumors. However, PCDGF expression was significantly higher in ER/PR-negative tumors. Concerning the correlation with p53, a larger percentage of tumors that expressed PCDGF with a staining intensity of 2+ or 3+ were p53-positive

(44%) than PCDGF-negative tumors (25%), $p<0.05$. Interestingly, PCDGF expression in IDC was independent of c-erbB-2 overexpression. 58.5% of tumors that stained for PCDGF with 2+ or 3+ intensity were c-erbB-2-negative. These studies provide the first direct evidence of high incidence of PCDGF expression in human breast cancer in which it correlates with clinico-pathological variables such as tumor type, tumor grade, steroid receptor status, proliferation index (determined by measuring Ki67 index) and p53 expression. These characteristics as well as the absence of expression in benign breast tissue suggest an important role of PCDGF in breast cancer pathogenesis and make it a potential novel target for the treatment of breast cancer. *Details of these studies are found in the appendix in the manuscript entitled "Expression of PC-Cell Derived Growth Factor (PCDGF) in benign and malignant human breast epithelium by Ginette Serrero and Olga Ioffe (submitted for publication).*

The difficulty encountered in conducting this study has been the time spent in locating tumor banks able to provide large numbers of samples outside of the University of Maryland. This is a problem experienced by many investigators engaged in biomarker research. This problem was identified and discussed on many occasions at the latest Era of Hope in the interactive sessions and town meeting. Support for nationwide-shared resources for this type of studies will be welcome by the cancer research community.

Studies are on-going now to correlate PCDGF expression in invasive breast carcinomas with metastasis and with response to therapy.

KEY RESEARCH ACCOMPLISHMENTS:

1. Develop an anti human PCDGF antibody that can be used for immunohistochemistry in paraffin embedded sections.
2. Develop and optimized staining conditions for IHC determination of PCDGF expression using anti-PCDGF antibody.
3. Secured and collected 206 samples for our study using two tumor Banks one for the University of Maryland Cancer Center and from the Manitoba Breast Cancer tumor Bank. For both sets of samples, PI obtained clinical parameters for future correlation studies. 5 slides were obtained for each case in order to carry out staining for PCDGF, ER/PR, erbB2, p53 and ki 67.
4. Performed and scored PCDGF staining of the 206 samples
5. Read and scored all the slides for each marker with Dr. Olga Ioffe Breast Pathologist at the University of Maryland Cancer Center.
6. Prepared a database of the results and carry out correlation studies of PCDGF expression for each marker and with clinical parameters.
7. Carry out statistical analysis of the data.
8. Prepared and submitted a manuscript describing the studies and the results obtained.
9. On-going: Investigate the relationship between PCDGF overexpression and acquisition of tamoxifen resistance in human breast cancer cell lines.

REPORTABLE OUTCOMES:

Manuscript submitted:

Serrero, G and Ioffe, O.B. (2002) Expression of PC-Cell Derived Growth Factor (PCDGF) in benign and malignant human breast epithelium. Submitted

Abstracts and presentations:

1. Abstract for poster presentation

AACR Annual Meeting San Francisco CA, April 6-10 2002
Tangkeangsirisin, W, Chen X, Dai, H and Serrero, G (2002) Role of PC-Cell Derived Growth Factor in the alteration of estrogen responsiveness and in the acquisition of anti-estrogen resistance in human breast cancer.
This abstract was selected for a Scholar-in-Training Award presented to Wisit Tankeangsirisin graduate student that presented his work at the Annual Meeting of AACR in San Francisco April 2002.

2. Abstract for invited oral presentation

Susan G. Komen Mission conference. June 2002 Washington DC
Ginette Serrero, Identification of a Novel Therapeutic Target for Human Breast Cancer

3. Abstract for invited poster presentation

Era of Hope DOD Conference Orlando FL September 2002
Serrero, G and Ioffe O. (2002) IHC study of a novel molecular marker of infiltrating ductal carcinoma.

CONCLUSION:

Our studies with human breast cancer cell lines have indicated the biological importance of PCDGF in breast cancer tumorigenesis (Lu and Serrero, 2000;2001), thereby warranting the investigation of its expression in archived pathological samples. This is the first report describing the expression of PCDGF in human breast tissue. We have shown that PCDGF is not expressed in benign breast epithelium. PCDGF expression was not seen in lobular carcinoma in situ, whereas the majority of invasive lobular carcinoma cases (82%) were either negative or weakly positive. PCDGF expression was found in most cases of in situ and invasive ductal carcinoma. PCDGF was expressed in 80% of IDC with a staining of 2+ or greater in 41% of the cases. In malignant ductal lesions, the degree of PCDGF staining correlated with the histologic grade in DCIS and IDC and with the proliferation (Ki-67) index. We have shown here that PCDGF expression correlates with estrogen and progesterone receptor status in invasive carcinomas, with ER+/PR+ cases having lower PCDGF expression than ER^-/PR^- tumors. Our study showed that PCDGF expression correlates with p53 immunoreactivity, which is an accepted indicator of the presence of p53 mutation. p53 positivity has been shown to be associated with poor outcome, especially in lymph node-negative breast cancer (18, 19), and it is an independent prognostic marker. In summary, our studies provide the first direct evidence of high incidence of PCDGF expression in human breast cancer, in which it correlates with such clinicopathological variables as tumor grade, proliferation index, steroid receptor expression and p53 expression. These characteristics,

along with the lack of expression in benign epithelium, and considering our previous studies in breast cancer cell lines suggest the important role of PCDGF in breast cancer and make it a potential target for the development of novel therapy for the treatment of breast cancer. We are also investigating whether PCDGF expression in pathological samples can be used as a predictor to response to therapy thereby making it an important and novel biomarker for breast cancer diagnosis.

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Lu, R and Serrero, G. (1999) Stimulation of PC cell derived growth factor expression by estradiol in human breast carcinoma cell line MCF-7. Biochem Biophys Res. Commun. 256, 204-207.

Lu, R and Serrero, G (2000) Inhibition of PC-cell derived growth factor expression inhibits tumorigenicity of the human breast carcinoma cell line MDA-MB-468 cells. Proc. Natl. Acad. Sci USA. In press.

Lu, R and Serrero, G. (2001) Mediation of estrogen mitogenic activity by PC-Cell derived growth factor in human breast cancer cells. Submitted to publication.

APPENDIX:

Manuscript *"Expression of PC-Cell Derived Growth Factor (PCDGF) in benign and malignant human breast epithelium by Ginette Serrero and Olga Ioffe (submitted for publication).*

Expression of PC-Cell Derived Growth Factor (PCDGF) in benign and malignant human breast epithelium

Ginette Serrero[1*] and Olga B. Ioffe[2]

1: Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy and Program in Oncology Marlene and Stewart Greenebaum Cancer Center of the University of Maryland. 2: Department of Pathology, University of Maryland School of Medicine. Baltimore, MD 21201

*: Corresponding Author:
Dr. Ginette Serrero
Department of Pharmaceutical Sciences
University of Maryland School of Pharmacy
20 N. Pine St.
Baltimore, MD 21201-1180
Phone: 410 706-6639
Fax: 410 706-0346
Email: gserrero@rx.umaryland.edu

Running Title: PCDGF in human breast tissue

Key Words: PC-derived growth factor, breast cancer, immunohistochemistry, prognostic markers, Ki-67, p53[1]

[1] Abbreviations: PCDGF – PC cell derived growth factor, IDC – invasive ductal carcinoma, DCIS – ductal carcinoma in situ, ILC – invasive lobular carcinoma, LCIS – lobular carcinoma in situ, ER – estrogen receptor, PR – progesterone receptor.

ABSTRACT

PC cell derived growth factor (PCDGF, also known as progranulin) is a novel autocrine growth factor shown to be overexpressed and to be mitogenic in human breast cancer cell lines. Inhibition of PCDGF expression in human breast carcinoma was associated with inhibition of tumorigenesis in vivo. In the present study, we have examined PCDGF expression by immunohistochemistry in paraffin embedded human breast tissue and investigated its association with clinicopathological variables. The study included 206 human breast lesions and has shown that PCDGF staining is observed in breast carcinoma whereas it is negative in benign breast epithelium. Analysis of various types of breast cancer indicated that PCDGF was predominantly expressed in invasive ductal carcinoma rather than in invasive lobular carcinoma. 80% of invasive ductal carcinomas were positive for PCDGF with 41% of cases staining with at least 2+ intensity. In contrast, 47% of invasive lobular carcinomas were negative for PCDGF, and only 18% of positive tumors showed at least 2+ reactivity. No PCDGF staining was observed in lobular carcinoma in situ. Ductal carcinoma in situ expressed PCDGF in 66% of the cases, and this expression correlated strongly with the nuclear grade of DCIS. Similar correlation was observed between the degree of PCDGF expression and histologic grade of invasive ductal carcinoma. Correlation studies were carried out in invasive cancers between PCDGF staining intensity and c-erbB-2, estrogen (ER), progesterone (PR) and p53 expression, and the proliferation rate expressed by Ki-67 index. The latter index was proportional to the degree of PCDGF expression in the tumor cells, with the average Ki-67 index of PCDGF-negative/weakly positive tumors (30.3) significantly lower than that of strongly PCDGF-positive carcinomas (48.8, $p<0.05$).

PCDGF was expressed in both ER/PR-positive and negative tumors. However, PCDGF expression was significantly higher in ER/PR-negative tumors. Concerning the correlation with p53, a larger percentage of tumors that expressed PCDGF with a staining intensity of 2+ or 3+ were p53-positive (44%) than PCDGF-negative tumors (25%), $p<0.05$. Interestingly, PCDGF expression in IDC was independent of c-erbB-2 overexpression. 58.5% of tumors that stained for PCDGF with 2+ or 3+ intensity were c-erbB-2-negative.

Our studies provide the first direct evidence of high incidence of PCDGF expression in human breast cancer in which it correlates with clinicopathological variables such as tumor grade, steroid receptor status, proliferation index and p53 expression. These characteristics as well as the absence of expression in benign breast tissue suggest an important role of PCDGF in breast cancer pathogenesis and make it a potential novel target for the treatment of breast cancer.

INTRODUCTION

PC cell derived growth factor (PCDGF) is an 88-kDa glycoprotein originally purified from the highly tumorigenic mouse teratoma-derived cell line PC (1, 2). PCDGF (also known as progranulin) is the largest member of a novel family of cysteine-rich polypeptides that include the 6 kDa epithelins or granulins shown to either promote or inhibit cell growth depending on the cell type tested (3, 4). Our laboratory was the first to demonstrate the biological activity of PCDGF as a growth promoter for the tumorigenic teratoma PC cells (2). Others later demonstrated growth-promoting activity of the precursor for other mesenchymal and epithelial cells as well as for pre-implantation embryos (5-7).

Screening of human tumor cell lines for PCDGF expression indicated that it was highly expressed in estrogen receptor-negative (ER^-) human breast carcinomas whereas it was barely detectable in the non-tumorigenic immortalized mammary epithelial cells (8). Inhibition of PCDGF expression by antisense PCDGF cDNA transfection in ER^- human breast carcinoma resulted in a dramatic reduction (more than 98%) in tumor incidence and tumor size when injected in nude mice (8), implicating PCDGF as a major factor in the maintenance of tumor phenotype. In ER-positive (ER^+) cells such as MCF-7 and T47D, PCDGF expression was transcriptionally stimulated by 17-β estradiol (E2) and inhibited by the anti-estrogen tamoxifen (9). Recently, we demonstrated that PCDGF mediated E2 mitogenic effect in ER^+ breast cancer cells (10). Importantly, overexpression of PCDGF in MCF-7 cells rendered the cells able to proliferate in the absence of estrogen although estrogen receptor expression of the cells remained unchanged (10). These various studies pointed out that PCDGF was important for the

proliferation of breast cancer cells and that increase of PCDGF expression played a major role in the maintenance of the breast cancer phenotype. Based on these observations, the present study was carried out to investigate PCDGF expression in human breast cancer. PCDGF expression was determined by immunohistochemical staining using an anti-human PCDGF antibody in formalin-fixed, paraffin-embedded benign and malignant human breast tissue. In addition, PCDGF expression was also examined in association with clinicopathological variables such as the histologic grade and type, expression of estrogen and progesterone receptors (ER/PR) as well as other markers such as proliferation rate (Ki67 index), p53 and c-erbB-2, known for their prognostic value in breast cancer.

Ki-67 (MIB-1) is a protein vital to cell proliferation (11); it is a nuclear antigen expressed in all phases of the cell cycle except in G1 or G_0 phase (12), and is recognized by the antibody MIB-1 in paraffin-embedded archival tissue. High Ki-67 index has been shown to correlate with shortened disease-free survival in breast cancer on multivariate analysis (13), and with shortened overall and disease-free survival (14).

c-erbB-2 (HER2/neu) is an oncogene that encodes a 185-kDa ligandless receptor tyrosine kinase belonging to the epidermal growth factor receptor superfamily. Its overexpression (seen in 20-30% of breast cancers) has been linked to poor outcome, especially in node-positive patients (15), whereas its effect on prognosis in node-negative disease has remained controversial (reviewed in (16, 17)).

p53 is a tumor suppressor gene involved in cell cycle arrest. In breast cancer, it detects high-risk patients, especially if they are node-negative. p53 mutations, which can

be identified immunohistochemically, are linked to poor prognosis, high histologic grade and proliferation rate, aneuploidy and steroid receptor negativity (18, 19).

MATERIAL AND METHODS

Tissue samples.

Two hundred and six nonconsecutive archival formalin-fixed, paraffin-embedded human breast tissue samples from 152 patients were obtained from the University of Maryland Department of Pathology files and from the NCIC-Manitoba Breast Cancer Tumor Bank of the University of Manitoba, Winnipeg Canada (kindly provided by Dr. Peter Watson). The cases examined included 27 ductal carcinoma in situ (DCIS), 12 lobular carcinoma in situ (LCIS), 124 invasive ductal carcinoma (IDC), 17 invasive lobular carcinoma (ILC), and 26 benign breast tissue. The DCIS cases were graded according to nuclear grade. The Elston (Nottingham) grading system (20) was used for the determination of histologic grade of IDC. Steroid hormone receptor status was available for most of invasive carcinoma cases.

Immunohistochemistry

Four-micrometer sections were cut from a representative paraffin block in each case; these sections were immunostained using a standard peroxidase-conjugated streptavidin biotin method. The tissue sections were dewaxed and then rehydrated. Antigen retrieval was performed using DAKO target retrieval solution (DAKO corporation, Carpenteria, CA). Immunostaining was performed using Ventana autostainer

(Ventana, Tucson, AZ). The slides were counterstained with hematoxylin. Appropriate positive and negative controls were included in each run.

Detection of PCDGF by immunohistochemistry

PCDGF was detected in tissue sections by immunostaining using an immunoaffinity purified anti-human PCDGF antibody (1 μg/ml). Purity and specificity of the antibody had been previously determined by SDS-PAGE and western blot analysis. On western blot analysis of cell lysates, this antibody recognized a single 88 kDa band that could be competed by pre-incubation of the antibody with excess antigen as described previously (10). PCDGF expression was semiquantitatively categorized as follows: <5% of cells staining – negative, >5% of cells staining – positive; positive staining was graded from weak/focal (1+) to moderate/focal or diffuse (2+) to strong/diffuse (3+).

Detection of Ki-67, p53 and c-erbB2

The proliferation rate was measured by determining the Ki-67 index. Detection of Ki67 was carried out by immunostaining using MIB-1 antibody (DAKO corporation, Carpenteria, CA). Ki67 index was expressed as the percentage of positively staining nuclei per 1000 cells counted. Immunostaining for p53 was performed by using an anti-p53 antibody from BioGenex Laboratories (San Ramon, CA). Expression of p53 was categorized as follows: <5% of nuclei staining – negative; >5% of nuclei staining – positive. c-erbB-2 immunostaining was performed using anti-c-erbB-2 antibody (DAKO corporation, Carpenteria, CA). c-erbB-2 expression was assessed by the presence and

intensity of the cell membrane staining as follows: <10% staining – negative; >10% cells staining – positive; positive staining was graded from focal, weak, discontinuous membrane staining (1+) to focal moderate continuous membrane reactivity (2+), to homogeneous strong and continuous membrane positivity (3+).

Statistical analysis of the data

Statistical analysis of the data was performed using the ANOVA method, F-test for variances, regression test and chi-square test.

RESULTS

Histopathologic composition of the cases included in the study

The cases examined included 27 ductal carcinoma in situ (DCIS), 12 lobular carcinoma in situ (LCIS), 124 invasive ductal carcinoma (IDC), 17 invasive lobular carcinoma (ILC), and 26 benign breast tissue. The composition, histologic grade and type of the cases examined in this study are shown in Table 1.

PCDGF expression in human breast tissue and its association with histologic type

PCDGF expression was observed in 128 of all 206 cases examined (62%) (Table 2). Most benign breast epithelium was negative for PCDGF (25 out of 26 cases, or 96%), as were most LCIS cases (11 of 12, or 92%). However, the majority of malignant non-invasive and invasive ductal lesions showed PCDGF expression. Eighteen of the 27 DCIS (67%) and even a higher proportion of IDC, 99 of 124 (79.8%), expressed PCDGF. ILC expressed PCDGF in about half of the cases – 9 of 17 (53%). There was a striking

difference in the degree of PCDGF expression between invasive ductal and lobular carcinomas. While 51 of 124 (41%) cases of IDC showed moderate or strong PCDGF reactivity (2+ or 3+), only 3 of 17 (18%) ILC were moderately or strongly positive ($p<0.05$, chi-square test). Overall, the differences in PCDGF staining between benign tissue/LCIS, ILC and malignant ductal lesions were statistically significant ($p<0.05$). Figure 1A shows strong (3+) PCDGF staining in IDC. The staining was confined to the tumor cell cytoplasm; the character of staining was coarsely to finely granular. Note a PCDGF-negative benign duct surrounded by infiltrating carcinoma. The typical absence of PCDGF expression in in situ and invasive lobular carcinomas is shown in Figures 1B and 1C.

PCDGF expression and histologic grade of ductal carcinoma

As shown in Tables 3 and 4, PCDGF expression showed a strong correlation with the histologic grade of both in situ and invasive ductal carcinomas. While the majority of low nuclear grade DCIS were negative for PCDGF (9 of 16, or 56%) and all the remaining cases were weakly (1+) positive, all DCIS of intermediate nuclear grade (3 of 3, or 100%) and most high-grade DCIS (7 of 8, or 87.5%) showed moderate/high PCDGF positivity (Fig. 1D). It is important to note that all DCIS of intermediate or high nuclear grade were positive for PCDGF. The difference in PCDGF expression between low and intermediate/high grade DCIS was statistically significant ($p<0.05$).

A similar pattern of PCDGF distribution in relation to histologic grade was seen in IDC. All grade 1 tumors were either negative or weakly positive for PCDGF: 20 of 36 (55.5%) were negative, and the remaining 16 of 36 (44.5%) weakly positive. In contrast,

almost half of grade 2 tumors showed moderate to strong PCDGF reactivity (19 of 40, or 47.5%), and only 5 (12.%) IDC of grade 2 were negative. Moreover, none of the 48 grade 3 carcinomas lacked PCDGF expression, and two-thirds (32 of 48, or 67%) showed moderate to strong immunoreactivity for PCDGF. The differences in PCDGF staining between IDC tumors of all three histologic grades were statistically significant ($p<0.05$).

PCDGF expression and proliferation rate of invasive carcinomas

The proliferative rate as expressed by Ki-67 index showed a significant correlation with PCDGF expression in all invasive carcinomas (Figure 2). The average Ki-67 index of PCDGF-negative carcinomas was 30.3. The Ki-67 index was 32.7 in weakly positive tumors, 34.8 in cancers that were moderately (2+) PCDGF-positive and 48.8 in strongly (3+) PCDGF-positive tumors. The Ki-67 index of the tumors that were either negative or weakly positive was significantly lower than that of the moderately/strongly PCDGF-positive carcinomas, $p=0.01$.

Correlation of PCDGF expression and ER/PR status

Of 141 invasive carcinomas for which the ER and PR status was available, 57 (40.4 %) were positive for both ER and PR. Forty tumors (28.4%) were positive for ER and negative for PR, and 44 (31.2%) were negative for both receptors. No cases negative for ER and positive for PR (ER^-/PR^+) were seen in this study. It has been previously reported that ER^-/PR^+ tumors are relatively rare, accounting for only 4% of all breast cancers (21). The ER/PR status was compared to the PCDGF expression of the same invasive tumors (Table 5). Of the 86 invasive cancers that were negative or weakly

positive for PCDGF expression, 42 (48.8%) were positive for ER and PR, compared with 15 of 55 (27.3%) PCDGF-expressing cancers that were ER/PR-positive ($p<0.002$). Interestingly, the ER^+/PR^- tumors were similar in their PCDGF expression to the ER/PR-negative carcinomas, with 21 of 40 (52.5%) ER^+/PR^- cases and 19 of 44 (43.2%) of ER/PR-negative tumors were moderately to strongly PCDGF-positive.

PCDGF expression and p53 status

Among the 73 cases of invasive carcinomas that were negative or weakly positive for PCDGF, 55 of 73 (75%) were negative for p53, and 18 of 73 (25%) were p53-positive. p53 expression was significantly more common in IDC that strongly expressed PCDGF. Of the 50 PCDGF-expressing (2+/3+) cases, 22 (44%) exhibited p53 reactivity. Therefore, a larger percentage of tumors that expressed PCDGF with a staining intensity of +2 or + 3 were p53 positive when compared to PCDGF-negative tumors, $p=0.02$ (Table 6).

PCDGF expression and c-erbB-2 overexpression

Among the cases of invasive carcinoma that were negative or weakly positive for PCDGF, 46 of 80 (57.5%) were negative or weakly positive (1+) for c-erbB-2, 25 of 80 (31.3%) were moderately (2+) positive for c-erbB-2, and 19 of 80 cases (23.8%) were strongly c-erbB-2-positive (3+) (Table 7). Of the cases moderately to strongly expressing PCDGF (2+/3+), 31 out of 53 (58.5%) did not show immunohistochemical evidence of c-erbB-2 overexpression (0/1+ c-erbB-2 staining), and 13 of 53 cases (24.5%) had strong c-

erbB-2 reactivity (3+). These results indicate that there is no correlation between c-erbB-2 and PCDGF expression in the invasive mammary carcinomas examined.

DISCUSSION

Our studies with human breast cancer cell lines have indicated the biological importance of PCDGF in breast cancer tumorigenesis (8-10), thereby warranting the investigation of its expression in archived pathological samples. This is the first report describing the expression of PCDGF in human breast tissue. We have shown that PCDGF is not expressed in benign breast epithelium. PCDGF expression was not seen in lobular carcinoma in situ, whereas the majority of invasive lobular carcinoma cases (82%) were either negative or weakly positive. PCDGF expression was found in most cases of in situ and invasive ductal carcinoma. PCDGF was expressed in 80% of IDC with a staining of 2+ or greater in 41% of the cases. Therefore, there appears to be a preferential expression of PCDGF in ductal rather than lobular lesions. In malignant ductal lesions, the degree of PCDGF staining correlated with the histologic grade in DCIS and IDC. These data support the fact that in human breast cancer cell lines, the level of PCDGF expression in breast carcinoma cell lines appear to be positively correlated to their tumorigenesis (8). Interestingly, PCDGF staining intensity in invasive carcinomas correlated with the proliferation (Ki-67) index. Growth fraction determined by Ki67 in breast carcinomas closely correlates with many others indicators of proliferation, such as S-phase by flow cytometry, (22) thymidine labeling index (23), BrdU index (24, 25), and mitotic counts (25). The fact that PCDGF expression correlated with Ki67 index is important since Ki-67 index is an independent factor of poor prognosis, especially in node-negative patients

(14, 24). We have previously shown that PCDGF is a growth stimulator of several breast cancer cell lines and that inhibition of PCDGF expression by antisense transfection or action by neutralizing antibodies resulted in inhibition of in vitro cell proliferation and in vivo tumorigenesis (8, 10). The finding that PCDGF staining in the current study was significantly associated with Ki-67 index is in agreement with the fact that PCDGF is a growth stimulatory factor for breast cancer.

The correlation of PCDGF expression with histologic grade most likely stems from the growth-stimulating properties of PCDGF since it has been shown that the proliferative rates correlate with histologic grade of invasive and in situ ductal carcinomas (26-28).

We have shown here that PCDGF expression correlates with estrogen and progesterone receptor status in invasive carcinomas, with ER+/PR+ cases having lower PCDGF expression than ER^-/PR^- tumors. The fact that PCDGF expression is higher in the ER^+/PR^- than in ER^+/PR^+ cases and is similar to PCDGF expression in ER^-/PR^- tumors, supports the findings by McGuire et al (21), which showed that ER^+/PR^- breast carcinomas had low response rates to hormonal treatment, akin to ER-/PR- cancers. In their study, McGuire et al. postulated that PR-negativity might be an indicator of a false-positive estrogen receptor status. Our findings lend further support to this hypothesis.

It is interesting that both ER/PR status and proliferative rate show correlation with PCDGF expression. It has been shown that ER/PR-negative tumors tend to have higher proliferation rates (29). Our data would suggest that PCDGF contributes to the stimulation of proliferation in steroid receptor-negative tumors to a greater extent than in receptor-positive cancers.

Our study showed that PCDGF expression correlates with p53 immunoreactivity, which is an accepted indicator of the presence of p53 mutation. p53 positivity has been shown to be associated with poor outcome, especially in lymph node-negative breast cancer (18, 19), and it is an independent prognostic marker. Interestingly, p53 has been shown to directly correlate with Ki-67 index, and not with c-erbB-2 status (30), similar to PCDGF in our study.

Since PCDGF and c-erbB-2 are both implicated in the activation of growth promoting signaling pathways, the lack of significant association between PCDGF and c-erbB-2 in the cases studied here is interesting. c-erbB-2 overexpression, often the result of gene amplification, is associated with increased tumor growth rate, enhanced metastatic rate and shorter disease-free and overall survival rate (2, 16, 31, 32), although c-erbB-2 status has not been consistently proven to represent an independent prognostic indicator (33). The lack of correlation between PCDGF and c-erbB-2 expression is in agreement with results of our previous studies of human breast cancer cell lines, in which we have shown that high PCDGF expression could be observed in cells that did not express c-erbB-2 (MDA-MB-468 cells) as well as in cells reported to express c-erbB-2 such as MDA-MB-453 (10). The fact that PCDGF is overexpressed in IDC that are negative or weakly positive for c-erbB-2 would suggest that these two growth factor signaling pathways might be distinct and activated independently from each other. This possibility is presently investigated in detail in our laboratory.

Co-expression of epidermal growth factor (EGF) receptors and transforming growth factor alpha (TGF-alpha) has been shown to constitute an adverse prognostic feature for breast cancer patients (34, 35). Although no correlation was found between

PCDGF and c-erbB-2 expression, it should be pointed out that 24.5% of the cases examined were strongly positive for both PCDGF and c-erbB-2. Experiments are currently underway to investigate the prognostic significance of PCDGF expression either alone or in combination with c-erbB-2 in breast cancer.

The difference in PCDGF expression in invasive lobular and ductal carcinomas is of considerable interest. Although the prognosis of IDC and ILC is essentially identical if matched by stage (36, 37), the proliferation rates of ILC as measured by mitotic index, Ki-67 index, AgNOR measurement (24, 38) as well as S-phase fraction determination by flow cytometry (39) have been reported to be significantly lower than those of IDC. Since we have previously shown that PCDGF is a growth stimulator (8, 10), the relative lack of PCDGF expression in ILC certainly correlates with the low proliferation rates of these tumors. LCIS have been reported to have extremely low proliferation rates, (38, 40) similar to those of benign breast epithelium. These findings would certainly correlate with the lack of PCDGF expression in both benign breast epithelium and LCIS.

In summary, our studies provide the first direct evidence of high incidence of PCDGF expression in human breast cancer, in which it correlates with such clinicopathological variables as tumor grade, proliferation index, steroid receptor expression and p53 expression. These characteristics, along with the lack of expression in benign epithelium, and considering our previous studies in breast cancer cell lines suggest the important role of PCDGF in breast cancer and make it a potential target for the development of novel therapy for the treatment of breast cancer.

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Table 1. Cases included in the analysis

Diagnosis	# of cases
IDC	124
Grade 1	36 (29%)
Grade 2	40 (32.3%)
Grade 3	48 (38.7%)
ILC	17
DCIS	27
High nuclear grade	16 (59.3%)
Intermediate nuclear grade	3 (11%)
Low nuclear grade	8 (29.7%)
LCIS	12
Benign	26

IDC – invasive ductal carcinoma, ILC – invasive lobular carcinoma, DCIS – ductal carcinoma in situ, LCIS – lobular carcinoma in situ.

Table 2. Results of PCDGF immunostaining

Diagnosis	# of cases	PCDGF staining		
		Negative	Weak (1+)	Moderate/Strong (2+/3+)
Benign	26	25 (96%)	1 (4%)	0
DCIS	27	9 (33%)	8 (30%)	10 (37%)
LCIS	12	11 (92%)	1 (8%)	0
IDC	124	25 (20.2%)	48 (38.8%)	51 (41%)
ILC	17	8 (47%)	6 (35%)	3 (18%)

IDC – invasive ductal carcinoma, ILC – invasive lobular carcinoma, DCIS – ductal carcinoma in situ, LCIS – lobular carcinoma in situ. The differences in PCDGF expression between benign/LCIS, and intraductal/invasive carcinomas are statistically significant ($p<0.05$).

Table 3. PCDGF staining in ductal carcinoma in situ (DCIS)

Nuclear grade of DCIS	PCDGF staining		
	Negative (0)	Weak (1+)	Moderate/Strong (2+/3+)
Low grade, n=16	9 (56%)	7 (44%)	0
Intermediate grade, n=3	0	0	3 (100%)
High grade, n=8	0	1 (12.5%)	7 (87.5%)

Immunohistochemical expression of PCDGF significantly correlates with nuclear grade of DCIS ($p<0.05$).

Table 4: PCDGF staining in invasive ductal carcinoma (IDC) according to its histologic grade

IDC grade	PCDGF staining		
	Negative (0)	Weak (1+)	Moderate/Strong (2+/3+)
Grade 1, n=36	20 (55.5%)	16 (44.5%)	0
Grade 2, n=40	5 (12.5%)	16 (40%)	19 (47.5%)
Grade 3, n=48	0	16 (33%)	32 (67%)

PCDGF expression shows significant correlation with histologic grade of IDC ($p<0.05$).

Table 5. PCDGF expression and steroid receptor status in invasive carcinomas

PCDGF	Total #	ER+/PR+	ER+/PR-	ER-/PR+	ER-/PR-
Negative (0)	32	17 (53.1%)	6 (18.8%)	0	9 (28.1%)
Weak (1+)	54	25 (46.3%)	13 (24.1%)	0	16 (29.6%)
Moderate/Strong (2+/3+)	55	15 (27.3%)	21 (38.2%)	0	19 (34.5%)

Immunohistochemical expression of PCDGF significantly correlates with steroid receptor status ($p=0.03$).

Table 6. Comparison of PCDGF staining and p53 expression in invasive breast carcinoma.

PCDGF staining	Total #	p53 expression	
		Negative	Positive
Negative (0)	32	26 (81.3%)	6 (18.7%)
Weak (1+)	41	29 (70.7%)	12 (29.3%)
Moderate/Strong (2+/3+)	50	28 (56%)	22 (44%)

Immunohistochemical expression of PCDGF significantly correlates with p53 reactivity ($p < 0.05$).

Table 7: Comparison of PCDGF staining and c-erbB-2 expression in invasive breast carcinoma.

PCDGF staining	Total #	c-erbB-2 staining		
		0/1+	2+	3+
Negative (0)	29	18 (62.1%)	5 (17.2%)	6 (20.7%)
Weak (1+)	51	28 (54.9%)	10 (19.6%)	13 (25.5%)
Moderate/Strong (2+/3+)	53	31 (58.5%)	9 (17%)	13 (24.5%)

The immunohistochemical expression of PCDGF does not statistically correlate with c-erbB-2 status.

Figure legends

Figure 1. Immunostaining for PCDGF in paraffin- embedded human breast tissue. **A.** Strong (3+) diffuse cytoplasmic reactivity for PCDGF is seen in this invasive ductal carcinoma. A benign duct (arrow) surrounded by tumor is negative for PCDGF. **B.** Lobular carcinoma in situ shows no PCDGF expression. **C.** Invasive lobular carcinoma lacking PCDGF reactivity. Benign duct (arrow) is also negative for PCDGF. **D.** Ductal carcinoma in situ, high nuclear grade, solid type strongly expresses PCDGF (3+).

Figure 2. Box plot of Ki-67 index (% of positive cells) and PCDGF expression (graded from 0 to 3+) in invasive breast carcinomas. The difference between negative/weak and strong PCDGF expression is statistically significant ($p=0.01$).

Figure 1

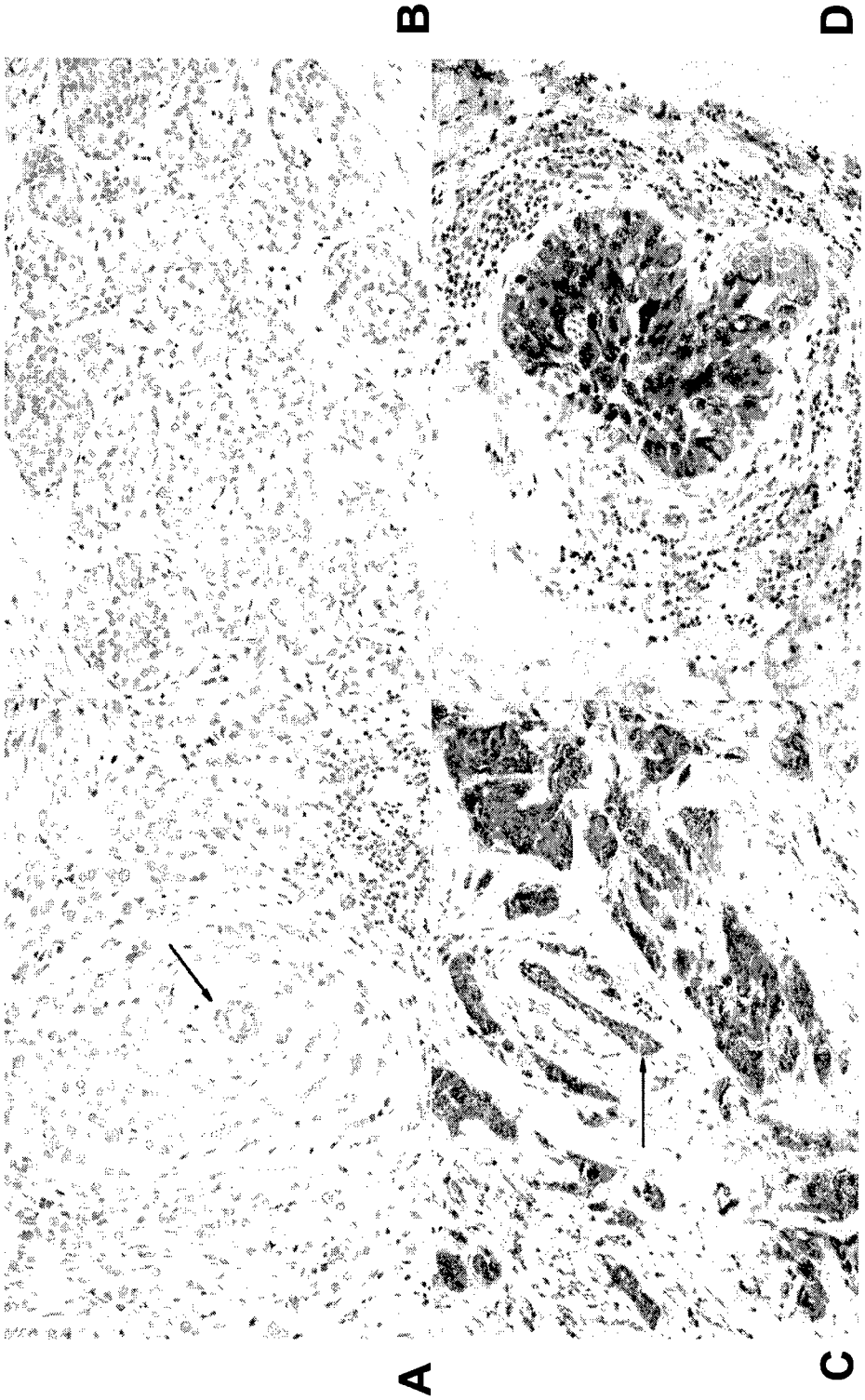
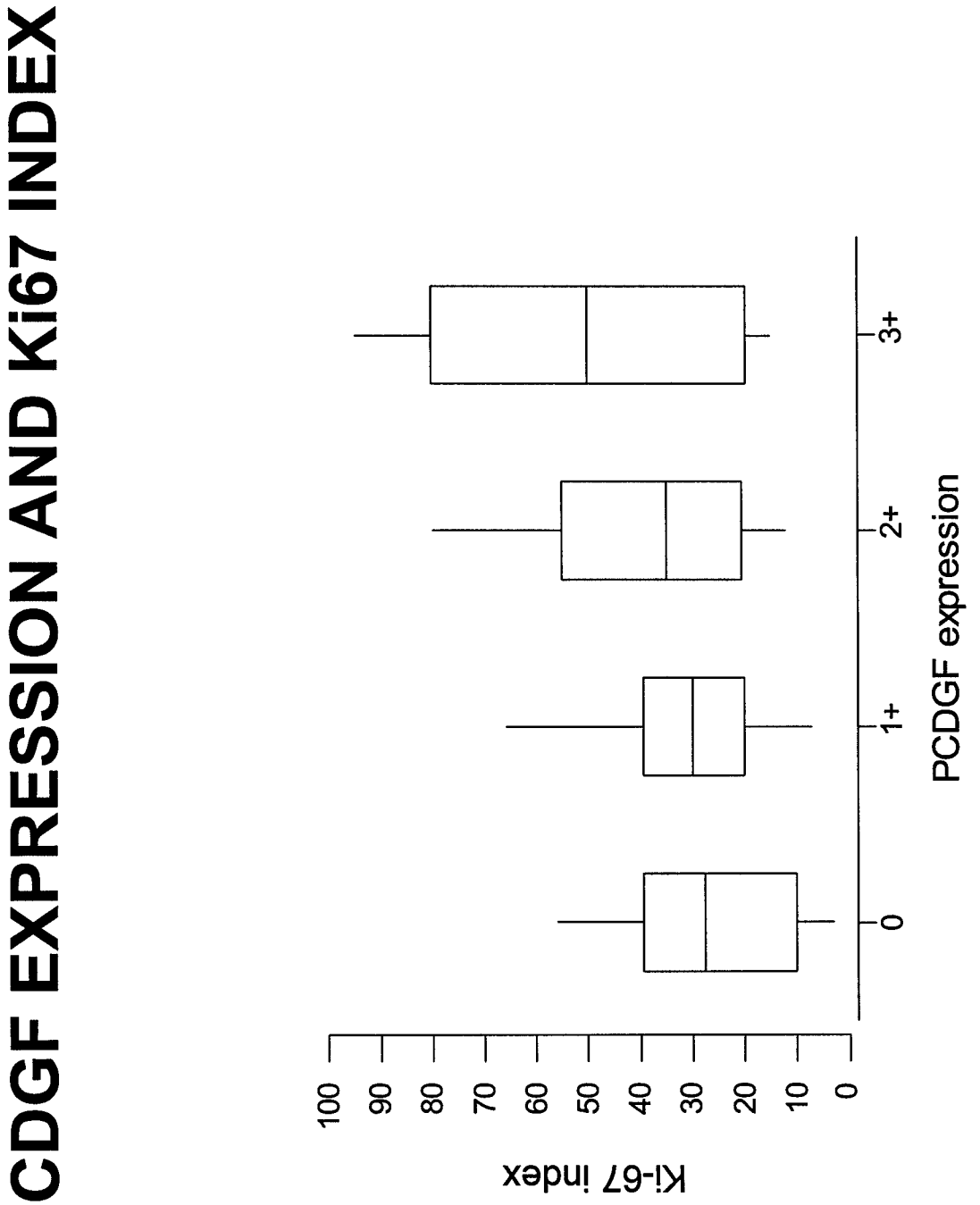


Figure 2


PCDGF EXPRESSION AND Ki67 INDEX
Ki-67 index
0
10
20
30
40
50
60
70
80
90
100
0
1+
2+
3+
PCDGF expression